

1 **Modulation of the inflammatory response of immune cells in human peripheral blood by**
2 **oxidized arachidonoyl aminophospholipids**

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Abstract

Aminophospholipids (APL), phosphatidylethanolamine (PE) and phosphatidylserine (PS), can be oxidized upon oxidative stress. Oxidized PE and PS have been detected in clinical samples of different pathologies and may act as modulators of the inflammatory response. However, few studies have focused on the effects of oxidized APL (ox-APL) esterified with arachidonic acid, even though a considerable number of studies have assessed the modulation of the immune system by oxidized 1-palmitoyl-2-arachidonoyl-*sn*-3-glycerophosphocholine (OxPAPC). In the present study, we have used flow cytometry to evaluate the ability of oxidized PAPE (OxPAPE) and PAPS (OxPAPS) to promote or suppress an inflammatory phenotype on monocytes subsets and myeloid dendritic cells (mDC). The results indicate that OxPAPE increases the frequency of all monocyte subpopulations expressing TNF- α , which promotes an inflammatory response. However, immune cell stimulation with OxPAPE in the presence of LPS results in a decrease of TNF- α expressed by classical monocytes. Incubation with OxPAPS and LPS induces a decrease in TNF- α produced by monocytes, and a significant decrease in IL-1 β expressed by monocytes and mDC, indicating that OxPAPS reduce the LPS-induced pro-inflammatory expression in these populations. These results show the importance of OxPAPE and OxPAPS as modulators of the inflammatory response and demonstrate their possible contribution to the onset and resolution of human diseases related to oxidative stress and inflammation.

Keywords: phosphatidylethanolamine, phosphatidylserine, lipid oxidation, lipidomics, flow cytometry, cytokines

List of abbreviations: 1,2-dipalmitoyl-*sn*-3-glycerophosphoethanolamine (DPPE), 1-palmitoyl-2-arachidonoyl-*sn*-3-glycerophosphocholine (PAPC), 1-palmitoyl-2-arachidonoyl-*sn*-3-glycerophosphoethanolamine (PAPE), 1-palmitoyl-2-linoleoyl-*sn*-3-glycerophosphoethanolamine (PLPE), 1-palmitoyl-2-oleoyl-*sn*-3-glycerophosphoethanolamine (POPE) 1-palmitoyl-2-arachidonoyl-*sn*-3-glycerophosphoserine (PAPS), aminophospholipid (APL), interleukin 1 beta (IL-

47 1 β), lipopolysaccharide (LPS), myeloid dendritic cell (mDC), phosphatidylethanolamine (PE),
48 phosphatidylserine (PS), tumour necrosis factor alpha (TNF- α).

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50 **Introduction**

51 Phosphatidylethanolamine (PE) and phosphatidylserine (PS), which are also termed
52 aminophospholipids (APL), are components of cell membranes, and display essential signaling
53 roles in several cellular processes [1]. PE is the second most abundant phospholipid class in cell
54 membranes and lipoproteins of mammalian organisms, contributing to 20 % of the whole
55 phospholipid profile [1], whereas PS is a less abundant class that constitutes 2-10% of total
56 phospholipids [2]. In mammalian cells, PE and PS contribute to membrane properties, such as
57 membrane curvature, fluidity, and impermeability to water and solutes. They are also mediators of
58 cell-cell interaction, and provide a membrane anchor for signaling macromolecules [1].

59 Upon oxidative stress, unsaturated fatty acyl chains and the polar head groups of APL can
60 be chemically modified, leading to the formation of oxidized PE and PS derivatives [3–5]. Several
61 reports have noted the possible interplay between oxidative stress, consequent oxidation of APL,
62 and inflammation. Oxidized PEs were detected in monocytes treated with IL-4 [6, 7], while
63 oxidized PS derivatives were found in mice treated with various external stressors, including
64 hyperoxia [8–10]. Oxidized PE and oxidized PS have also been detected in human pathologies, such
65 as cystic fibrosis [7] and Alzheimer’s disease [11], which share oxidative stress and inflammation
66 as common conditions underlying the onset of the disease [12, 13].

67 The formation of oxidized derivatives of PE and PS may shift the biological roles of the
68 native APL towards new functions. A role for oxidized PE in the development of atherosclerosis
69 was suggested by Zeiseniss *et al.*, who found oxidized 1-palmitoyl-2-linoleoyl-*sn*-3-
70 glycerophosphoethanolamine (OxPLPE) to be an active thrombogenic factor of oxidized low-

71 density lipoproteins [14]. More recently, oxidized derivatives of arachidonoyl-PE were highlighted
72 as the active components which mediate ferroptotic cell death [15]. Recent reports from our group
73 have also assessed the stimuli mediated by oxidized 1-palmitoyl-2-oleoyl-*sn*-3-
74 glycerophosphoethanolamine (OxPOPE), OxPLPE, glycated POPE and glycated PLPE in human
75 peripheral blood immune cells, highlighting the fact that all modified PE could induce a pro-
76 inflammatory response in the tested populations [16, 17].

77 Oxidized PS has been widely associated with apoptosis; several studies have highlighted its
78 role as a potent macrophage-activating factor [18–21], which was found to be a phagocytic signal
79 that, once externalized, mediates the engulfing of cells undergoing intrinsic apoptotic death [22].
80 Moreover, oxidized PS was found to induce vascular endothelium growth factor (VEGF) in two
81 types of human endothelium cells, which suggests a role in the progression and destabilization of
82 atherosclerotic plaques [23]. Silva *et al.* reported that oxidized 1-palmitoyl-2-oleoyl-3-*sn*-3-
83 glycerophosphoserine (OxPOPS), added directly to peripheral blood cells, induced a pro-
84 inflammatory phenotype in monocytes and dendritic cells [24].

85 Until now, few studies have focused on the role of PE and PS esterified with arachidonic
86 acid in the modulation of peripheral blood immune cells. In one report, Morgan *et al.* observed a
87 decrease in the production of cytokines in activated monocytes incubated with oxidized 1-stearoyl-
88 2-arachidonoyl-*sn*-3-glycerophosphoethanolamine (OxSAPE) [25]. In other studies, oxidized 1-
89 palmitoyl-2-arachidonoyl-*sn*-3-glycerophosphoserine (OxPAPS) was found to inhibit the
90 proliferation of T cells isolated from peripheral blood [26], and to antagonize the interaction
91 between lipopolysaccharide and soluble CD14 [27]. However, the majority of studies concerning
92 the effects of oxidized phospholipids on immunity used one phosphatidylcholine (PC) bearing an
93 esterified arachidonoyl chain, namely PAPC, as reviewed by Bochkov and co-authors [28, 29].
94 Oxidized PAPC (OxPAPC) was found to be a pro-inflammatory and immunogenic antigen in
95 apoptotic cells [30], and to activate TLR-4, with the consequent induction of IL-8 [31] and IL-6

96 [32]. 1-palmitoyl-2-oxovaleroyl-*sn*-glycerophosphocholine (POVPC), a short chain oxidation
97 product from OxPAPC, was found to induce the expression of TNF- α and IL-1 β in human
98 macrophages [33]. Other studies, however, have reported the ability of OxPAPC to antagonize the
99 interaction of bacterial endotoxins with TLRs and, thus, block their acute inflammatory responses,
100 including that induced by LPS [27, 34–36]. Knowing this rich interplay between OxPAPC
101 derivatives and the immune system, we can speculate that oxidized 1-palmitoyl-2-arachidonoyl-*sn*-
102 glycerophosphoethanolamine (OxPAPE) and OxPAPS species may interact with the immune cell
103 populations of peripheral blood, and, consequently, mediate the promotion or the resolution of an
104 inflammatory state.

105 Because of the lack of knowledge of the biological roles of the PE and PS congeners of
106 bioactive OxPAPC, in the present study we have obtained OxPAPE and OxPAPS by *in vitro*
107 oxidation, characterized them by mass spectrometry (MS), and added them to human peripheral
108 blood samples. Flow cytometry analysis was then carried out to assess the potential of OxPAPE and
109 OxPAPS in promoting or inhibiting an inflammatory phenotype in subpopulations of monocytes
110 and myeloid dendritic cells (mDC). Pro-inflammatory activities were evaluated in peripheral blood
111 cells stimulated in the absence of LPS, while anti-inflammatory activities were evaluated upon co-
112 incubation of blood cells with oxidized APL (ox-APL) and LPS. We report new insights into the
113 biological functions of oxidized arachidonoyl-aminophospholipid species in humans, in particular
114 in the modulation of the inflammatory response mediated by peripheral blood immune cells.

115 **Materials and Methods**

116 *Chemicals*

117 PAPE and PAPS were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).
118 FeCl₂ and H₂O₂ (30%, w/v) used for the Fenton reaction were acquired from Merck (Darmstadt,
119 Germany). Water was of MilliQ purity filtered through a 0.22 μ m filter (Millipore, USA). All

120 solvents used were HPLC grade. For cell stimulation, RPMI-1640 medium was purchased from
121 Gibco (Paisley, Scotland, UK); Brefeldin-A and lipopolysaccharide (LPS) from *Escherichia coli*
122 (serotype 055:B5) were from Sigma-Aldrich (St. Louis, MO, USA). For flow cytometry, the
123 conjugated monoclonal antibodies (mAbs) for HLA-DR V450 (clone L243), CD45 V500-C (clone
124 2D1), IL-1 β PE (clone AS10), CD33 APC (clone P67.6), and CD14 APCH7 (clone M ϕ P9) were
125 purchased from Becton Dickinson (BD, San Jose, CA, USA); and TNF- α FITC (clone MP6-XT22)
126 and CD16 PECy7 (clone 3G8) from BD Pharmingen (San Diego, CA, USA). Phosphate-buffered
127 saline (PBS) was purchased from Gibco, and the permeabilization kit, Intraprep, from Beckman
128 Coulter (Brea, CA, USA).

129 *Preparation of the ox-APL vesicles*

130 Vesicles of phospholipids were prepared in 5 mM ammonium bicarbonate buffer (pH 7.4).
131 For each oxidation experiment, 1 mg of phospholipid in chloroform was evaporated to dryness and
132 reconstituted in 446 μ L of buffer. The mixture was shaken mechanically on a vortex mixer for 10
133 minutes and sonicated for 1 minute. The oxidation was mediated by the hydroxyl radical generated
134 upon Fenton reaction conditions (Fe²⁺/H₂O₂). Hydroxyl radical is a partially reduced oxygen
135 species, characterized by its high reactivity, and is involved in lipid peroxidation *in vivo* [37]. The
136 oxidation was initiated by adding 4 μ L of FeCl₂ (5 mM stock solution) and 50 μ L of H₂O₂ (500 mM
137 stock solution) to the phospholipid/buffer mixture, giving final concentrations of 40 μ M FeCl₂ and
138 50 mM H₂O₂ in a final volume of 500 μ L. The mixture was incubated at 37 °C and 550 rpm, in the
139 dark, for 48 hours.

140 *Mass spectrometry*

141 The reactions of oxidation were monitored by electrospray-MS (ESI-MS) in a linear ion trap
142 mass spectrometer LXQ (ThermoFinnigan, San Jose, CA). An aliquot of 2 μ g of each sample was
143 diluted in methanol (2:400, v/v) and introduced through direct infusion in the mass spectrometer.

144 The LXQ linear ion trap mass spectrometer operated in the negative ion mode. Data acquisition and
145 analysis were performed using the Xcalibur Data System (V2.1, Thermo Fisher Scientific, USA).
146 The ESI-MS spectra of the oxidation reactions are available online as supplementary information.

147 *Samples*

148 A total of 6 peripheral blood (PB) samples from healthy adult subjects (1 male and 5
149 females; mean age: 47.4 ± 7.5 years old) were collected in heparin. Informed consent, in accordance
150 with the local ethics committee, was obtained from all the individuals enrolled in this study.

151 *In vitro stimulation of cytokine production by mDCs and monocytes*

152 For each individual under study, we prepared 10 tubes with 500 μ L of PB previously diluted
153 1:1 (v/v) in RPMI 1640 complete culture medium, supplemented with 2 mM l-glutamine. PB
154 immune cells were then subjected to 10 different experimental conditions: no stimulation (negative
155 control), stimulation with LPS (positive control), stimulation with each one of the following native
156 or modified phospholipids (PAPE, PAPS, OxPAPE, and OxPAPS), and stimulation with those
157 same phospholipids in the presence of LPS (PAPE+LPS, PAPS+LPS, OxPAPE+LPS, and
158 OxPAPS+LPS). For this, we added Brefeldin A (10 μ g/mL) from *Penicillium brefeldianum* (to
159 prevent the release of *de novo* produced cytokines outside the cells) to all tubes, 100 ng/mL of LPS
160 and/or 20 μ g/mL of phospholipids, to the corresponding stimulated tube. The samples were then
161 incubated at 37 °C, in a sterile environment with 5% CO₂ humidified atmosphere, for 6 hours.

162 *Flow cytometry analysis of cytokine production*

163 After the incubation period, the PB samples under the different experimental conditions
164 were stained with mAbs for membrane antigens (HLA-DR, CD45, CD16, CD33, and CD14),
165 incubated for 10 min at room temperature in the dark, washed once with 2 mL of PBS and
166 centrifuged for 5 min at 540g. The supernatant was discarded; cells were then fixed and
167 permeabilized with the Intrapep kit according to manufacturer's instructions, and stained with

168 mAbs for intracellular cytokines (TNF- α and IL-1 β). The samples were then incubated for 15 min at
169 room temperature in the dark, washed twice with 2 mL of PBS, resuspended in 500 μ L of PBS, and
170 immediately analysed in the flow cytometer.

171 *Data acquisition and analysis*

172 Data acquisition was performed with a FACSCantoTMII (BD) flow cytometer using
173 FACSDiva software (v6.1.2; BD). The whole sample from each tube was acquired and stored,
174 corresponding to a number of events always above 0.5x10.⁶ For data analysis, Infinicyt software
175 (version 1.7; Cytognos SL, Salamanca, Spain) was used.

176 *Immunophenotypic identification of classical, intermediate, and non-classical monocytes,* 177 *and mDCs*

178 The mAb panel used allowed the identification of classical, intermediate, and non-classical
179 monocytes, and mDCs, as follows: classical monocytes express high levels of CD14 in the absence
180 of CD16, together with high expression of CD33 and HLA-DR; intermediate monocytes present
181 high expression of CD14 together with an increased expression of CD16, and lower levels of CD33
182 compared to classical monocytes; finally, non-classical monocytes show CD16 positivity with a
183 decreased expression of CD14, the highest expression of CD45 and the lowest CD33 levels among
184 the three monocyte subpopulations, whereas HLA-DR expression is between that of classical and
185 intermediate monocytes. mDC are characterized by lower side-scatter light dispersion properties
186 and CD45 expression compared to monocytes, and high expression of CD33 and HLA-DR in the
187 absence of CD14 and CD16.

188 *Statistical methods*

189 Mean values and standard deviations, as well as median values and range, were calculated
190 for each variable under study by using the SPSS software program (SPSS 17.0, Chicago, USA). The
191 statistical significance of the differences observed between groups was evaluated using the paired-

sample Wilcoxon test. Differences between groups were considered statistically significant when p-value <0.05.

Results

The ability of the modified APL derivatives OxPAPE and OxPAPS to induce or inhibit the production of cytokines (TNF- α and IL-1 β) was evaluated in three subpopulations of peripheral blood monocytes (classical, intermediate and non-classical) and in mDC. To evaluate the effect of the modified APL, both the frequency of cytokine-producing cells, expressed as a percentage, and the total amount of cytokines produced per cell, expressed as mean fluorescence intensity (MFI), were determined for each tested condition.

We studied the production of the cytokines TNF- α and IL-1 β by classical, intermediate and non-classical monocytes and mDC in peripheral blood at basal level (negative control), upon stimulation with LPS (positive control), and after incubation with ox-APL (OxPAPE, OxPAPS) and non-modified APL (PAPE, PAPS). The results obtained from the incubation of peripheral blood with the different non-modified or ox-APL were compared with negative control, with the aim of seeing if these species would be able to induce an increase in either the frequency or amount of pro-inflammatory cytokines production by monocytes and mDC. Non-modified and ox-APL were also incubated with peripheral blood in the presence of LPS, to evaluate their ability to influence the immune cell function in an inflammatory environment. The results given by the co-incubation of blood with LPS and APL or ox-APL were compared with a positive control (LPS-treated blood) to see if these species could decrease the frequency or amount of cytokine production by monocytes and mDC. This allowed the assessment of the modulatory activities of the oxidized phospholipids studied, based on the different interactions of the APL with the antigen-presenting cells, with emphasis on the effect of oxidation and of the chemistry of the polar head group. The amount of ox-APL mixture chosen for each experiment (20 μ g) has already been optimized in other studies from our group [16, 17].

Identification of the oxidized species by direct infusion ESI-MS.

Analysis by direct infusion ESI-MS in negative ion mode was performed to identify the oxidized species present in the OxPAPE and OxPAPS mixtures obtained after oxidation of hydroxyl radical generated by the Fenton reaction. Comparing the ESI-MS spectra of the PAPE and PAPS standards before and after the Fenton reaction (Supplementary Figures 3 and 4, respectively), we were able to observe significant changes. For both PAPE and PAPS, new ions appeared at higher m/z values than the $[M-H]^-$ ions of the nonmodified species, and were identified as long chain oxidation products ($[M + nO]^-$, $n = 1-7$). The oxidation products identified in the OxPAPE and OxPAPS mixtures are summarized in Supplementary Table 3: the most abundant OxPAPE species was PAPE (+2O-4Da), followed by the hydroxy (PAPE+O) and hydroxy-hydroperoxy (PAPE+3O) derivatives. In the case of PAPS oxidation (Supplementary Figure 4; Table 3) the most abundant OxPAPS species were PAPS +4O, correspondent mainly to dihydroperoxy derivative, followed by the PAPS+3O (correspondent mainly to hydroxy-hydroperoxy derivative) and the polar head oxidation product bearing a terminal acetic acid (POPS-29Da) [4]. .

Evaluation of the activity of OxPAPE in monocytes and dendritic cells.

The frequency of cytokine-producing cells and the amount of cytokines produced per cell (measured as MFI) were tested in cells from peripheral blood characterized by basal immune parameters (negative control) and in peripheral blood after incubation with ox-APL. We did not observe any significant increase in the frequency of monocytes and mDC producing TNF- α and IL-1 β , after stimulation with OxPAPS compared with negative control. Also, incubation with OxPAPS compared with negative control did not significantly affect the amount of TNF- α or IL-1 β produced per cell, measured as MFI, in any of the populations tested (Table 1; Figure 1). Significant variations were only observed after incubation with OxPAPE. In this case, we saw a significant

241 increase in the frequency of cells expressing TNF- α , in all the monocyte subpopulations, after
242 stimulation with OxPAPE, compared with negative control (Table 1; Figure 1). A statistically
243 significant increase was also noted for classical monocytes expressing IL-1 β after stimulation with
244 OxPAPE compared with negative control (Table 1; Figure 1). Stimulation with OxPAPE did not
245 induce any significant increase in the frequency of intermediate monocytes, non-classical
246 monocytes expressing IL-1 β , or in the frequency of mDC expressing TNF- α and IL-1 β , when
247 compared with negative control. Stimulation with OxPAPE did not lead to significant changes in
248 the amount of TNF- α or IL-1 β produced per cell, measured as MFI, in any of the tested cell
249 populations. With the native PAPE and PAPS, we found a significant increase in the frequency of
250 classical monocytes and intermediate monocytes expressing TNF- α after stimulation with PAPE
251 compared with negative control (Supplementary Table 1; Supplementary Figure 1). However, after
252 stimulation with PAPS, we found a significant increase in the frequency of classical monocytes and
253 intermediate monocytes expressing TNF- α and IL-1 β , when compared with negative control
254 (Supplementary Table 1, Supplementary Figure 1). Though not reaching statistically significant
255 levels, PAPS also exhibits a tendency to increase the percentage of cells producing TNF- α and IL-
256 1 β in non-classical monocytes and mDC. It is interesting to note that this ability of PAPS to
257 transversally promote pro-inflammatory cytokine expression in distinct monocyte subsets and mDC
258 is lost in OxPAPS.

259 *Evaluation of the activity of OxPAPS in monocytes and dendritic cells.*

260 The frequency of cytokine-producing cells and the amount of cytokines produced per cell
261 were tested in cells from peripheral blood upon LPS-induced acute inflammation (positive control),
262 and in cells from peripheral blood after incubation with LPS and non-modified or ox-APL. This
263 allowed us to evaluate whether these species could inhibit the inflammatory stimulus of LPS, i.e. if
264 they had any anti-inflammatory activity.

265 Co-incubation of LPS and OxPAPE did not lead to any significant decrease in the frequency
266 of monocytes or of mDC producing TNF- α and IL-1 β . Considering the amount of cytokines
267 produced per cell, co-incubation of LPS and OxPAPE led to a statistically significant decrease in
268 the amount of TNF- α expressed by classical monocytes with respect to positive control, expressed
269 as MFI (Table 2; Figure 2). However, the amount of IL-1 β produced by classical monocytes, and
270 the amount of TNF- α and IL-1 β produced by intermediate monocytes, non-classical monocytes, and
271 mDC, did not decrease significantly upon treatment with LPS + OxPAPE, against positive control.

272 We found that the co-incubation of LPS and OxPAPS induced a statistically significant
273 decrease in the amount of TNF- α produced by classical monocytes and non-classical monocytes, in
274 comparison with positive control. Incubation of OxPAPS with blood treated with LPS also induced
275 a significant decrease in the amount of IL-1 β expressed by non-classical monocytes and mDC
276 (Table 2; Figure 2). The amount of TNF- α expressed in intermediate monocytes and mDC, and the
277 amount of IL-1 β expressed by intermediate monocytes and classical monocytes, did not decrease
278 significantly when compared with positive control. Moreover, we saw that co-incubation LPS +
279 OxPAPS, in comparison with positive control, led to a significant decrease in the frequency of
280 mDC producing TNF- α (Table 2; Figure 2). The frequency of IL-1 β -expressing mDC, along with
281 the frequency of monocytes expressing TNF- α and IL-1 β , were not affected by LPS + OxPAPS. In
282 the case of native PAPE and PAPS, co-incubation of LPS and PAPE did not lead to any significant
283 decrease either in the frequency of monocytes and mDC producing TNF- α or IL-1 β , or in the
284 amount of cytokines produced per cell (MFI) (Supplementary Figure 2, Supplementary Table 2).
285 Treatment with LPS+PAPS compared to the positive control, led to a statistically significant
286 decrease in the amount of TNF- α produced by all the monocytes subpopulations, expressed as MFI
287 (Supplementary Figure 2, Supplementary Table 2). Interestingly, there is a marked contrast between
288 the striking inhibitory effect of OxPAPS over TNF- α expression by mDC and the absence of effect
289 observed for native PAPS.

Discussion

Oxidized phospholipids are recognized as important players in both the immune response and the development of various pathologies [29]. With pathologies that are associated with an increased ROS production, such as Alzheimer's disease, cystic fibrosis and alcoholic liver disease, the occurrence of ox-APL could be a potential source of molecules involved in the triggering or in the resolution of the inflammatory state [38, 11, 7]. With the aim of shedding more light on the relationship between oxidized arachidonoyl-APL and inflammation, we have used flow cytometry to obtain new insights into the modulation of peripheral blood immune cells by OxPAPE and OxPAPS. We have evaluated the influence of OxPAPE and OxPAPS on the frequency of monocytes and mDC that produce cytokines, and have assessed the quantity of TNF- α and IL-1 β produced by each cell subpopulation.

Among the ox-APL in this study, only OxPAPE induced a pro-inflammatory response after incubation with immune cells. In this case, the pro-inflammatory action was always due to an increase in the proportion of monocytes producing TNF- α and IL-1 β (classical, intermediate and non-classical monocytes); no significant effect was observed in the amount of cytokines produced per monocyte, and no effect was observed on mDC. This suggests that when OxPAPE is generated *in vivo* upon oxidative stress, it can promote an inflammatory environment by increasing the percentage of monocytes that produce pro-inflammatory cytokines. Oxidized PE has already been highlighted as a pro-inflammatory mediator [16, 17]. However, the effects of OxPAPE on peripheral blood immune cells have never been studied; this is the first report of the pro-inflammatory activity of OxPAPE as a group of species able to stimulate monocytes in peripheral blood.

OxPAPE is not the first arachidonoyl phospholipid for which a pro-inflammatory activity was observed. Walton and co-authors [31] reported that OxPAPC could bind toll-like receptor 4 (TLR4), a known receptor of bacterial endotoxins which activates intracellular signalling pathways

315 leading to cytokine production. Interestingly, the authors found that the binding of OxPAPC to
316 TLR4 occurred through a CD14-independent mechanism, promoting the activation of TLR4
317 receptor. In another report, OxPAPC was seen to stimulate cytokine production in macrophages and
318 induce acute lung injury through a pathway that included TLR4 and the activation of the TRIF
319 adaptor protein [32]. It is worth noting that the expression of TLR4 is markedly lower in mDC than
320 in monocytes [39]. Hence, we suggest that the specific stimulation of OxPAPE could be due to its
321 activating binding to TLR4. Thus the activation of TLR4 would induce transcriptional factors, such
322 as NF- κ B and AP-1, that ultimately lead to the expression of pro-inflammatory cytokines (TNF- α
323 and IL-1 β) [40, 41].

324 Oxidized PS did not show pro-inflammatory effects. This is in agreement with previous
325 work from our group, where we noted that OxPOPS species did not induce any pro-inflammatory
326 shift in peripheral blood immune cells [24]. In contrast, OxPAPS reduced the LPS-induced pro-
327 inflammatory phenotype in classical monocytes, non-classical monocytes, and mDC, in the
328 presence of LPS. In all the immune cell populations studied, OxPAPS decreased the amount of
329 cytokines produced per cell. This anti-inflammatory effect is in agreement with published works in
330 which OxPAPC and OxPAPS were found to antagonize the recognition of LPS by the CD14-TLR4
331 complex at several steps, arbitrating a multi-hit inhibition of the LPS-induced inflammatory
332 response in monocytes [27]. OxPAPS, in particular, was found to form a covalent complex with
333 CD14, a co-receptor for TLR4, acting as a competitive inhibitor of the binding between LPS and
334 TLR4 [27]. In addition, the externalization of oxidized PS on the membrane surface provides a
335 proven immunosuppressive mechanism leading to cell clearance and resolution of inflammation
336 [19, 42–44]. In HL60 and Jurkat cells undergoing intrinsic apoptosis, oxidized PS externalized on
337 the plasma membrane was found to directly participate in the process of cell engulfment mediated
338 by professional phagocytes [22]. Greenberg *et al.* [18] highlighted the role of CD36 as the receptor
339 by which macrophages recognize OxPAPS. Since CD36 is also expressed in monocytes [45] and

mDC [46], we suggest that the anti-inflammatory effect that we observed for OxPAPS could be due to the activation of CD36, whose intracellular signalling in monocytes leads to the inactivation of NF- κ B, and to a decreased expression of pro-inflammatory cytokines, such as TNF- α and IL-1 β [47, 48]. Several studies have reported that apoptotic cells exposing oxidized PS are capable of initiating the transduction of signals in mDC that induces the downregulation of various inflammatory pathways via inhibition of NF- κ B and TLRs (reviewed in reference [49]). The OxPAPS tested in the present study might have activated such anti-inflammatory signaling in peripheral mDC, thus leading to the decreased production of cytokines per mDC that we observed. The non-oxidized PAPE and PAPS were also able to induce an inflammatory response in classical and intermediate monocytes, but this was less than the ox-APL. A similar trend was already observed for 1,2-dipalmitoyl-3-*sn*-glycerophosphoethanolamine (DPPE) and PLPE [17]. We also saw an anti-inflammatory shift induced by PAPS in all monocyte subpopulations, in agreement with recent studies implicating native PS as an anti-inflammatory factor [50, 51]. However, non-modified APLs are mainly confined in the inner leaflet, where they are kept by the action of specific flippases [52] and scramblases.[53–57], that hinder PE and PS from the signaling interaction with other cells. Oxidation of PE and PS is known to induce the externalization of the ox-APL on the outer leaflet of mammalian cell membranes [19, 25, 43], and, as a consequence, the oxidized PAPE and PAPS, rather than their native congeners, seem to be involved in signalling events resulting in the modulation of the immune system, mentioned earlier.

Conclusions

The results of this study clearly demonstrate that oxidized arachidonoyl-APL can modulate the immune system and the inflammatory response in peripheral blood immune cells. We emphasized the role of OxPAPE as a promoter of the inflammatory response in circulating immune cells that is capable of inducing an increased frequency of TNF- α - and IL-1 β -producing monocytes. In contrast, we found that OxPAPS is an anti-inflammatory agent in peripheral blood

immune cells treated with the LPS bacterial endotoxin, and is able to downregulate the amount of cytokines produced per cell in each monocyte subpopulation and in mDC. Peroxidation of PAPE and PAPS occurs in inflammatory diseases characterized by oxidative stress. Thus, OxPAPE and OxPAPS can be key players in either the triggering or resolution of the inflammatory state underlying the onset and the development of such pathologies. For future studies, the information provided by flow cytometry can be integrated with detailed structural characterization of the OxPAPE and OxPAPS molecular species detected in clinical samples. This combined approach could provide a clear relationship between the structure of the ox-APL and its activity on the immune system, finally allowing the identification and the validation of new biomarkers of immune-mediated inflammatory diseases.

Author contributions

MRM, PD, and AP conceived and designed the study. SC, CMS, PL, and TM performed the experiments and analyzed the data. The manuscript was written by SC. All authors have reviewed and approved the final paper.

Conflicts of interest

The authors have no competing financial interests to declare.

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600 **Figure 1. Pro-inflammatory effect of OxPAPE.** Frequency of cells producing TNF- α and IL-1 β ,
 601 among classical (CMo), intermediate (IMo) and non-classical (NCMo) monocytes, and mDC, in the
 602 absence of LPS (negative control) and after incubation with OxPAPE and OxPAPS. Differences
 603 with respect to negative control were considered statistically significant (*) when $p < 0.05$ (paired-
 604 sample Wilcoxon test). %, the percentage of positive cells.

605 **Figure 2. Suppression of the inflammatory effect induced by LPS mediated by OxPAPS.**
 606 Frequency of cells producing TNF- α and IL-1 β , among classical (CMo), intermediate (IMo) and
 607 non-classical (NCMo) monocytes, and mDC, after stimulation with LPS (positive control), LPS +
 608 OxPAPE, or LPS + OxPAPS (A). Amount of TNF- α and IL-1 β produced per cell (measured by
 609 MFI), after stimulation with LPS (positive control), LPS + OxPAPE, or LPS + OxPAPS (B).
 610 Differences with respect to positive control were considered statistically significant (*) when $p <$
 611 0.05 (paired-sample Wilcoxon test). %, the percentage of positive cells. MFI, mean fluorescence
 612 intensity.

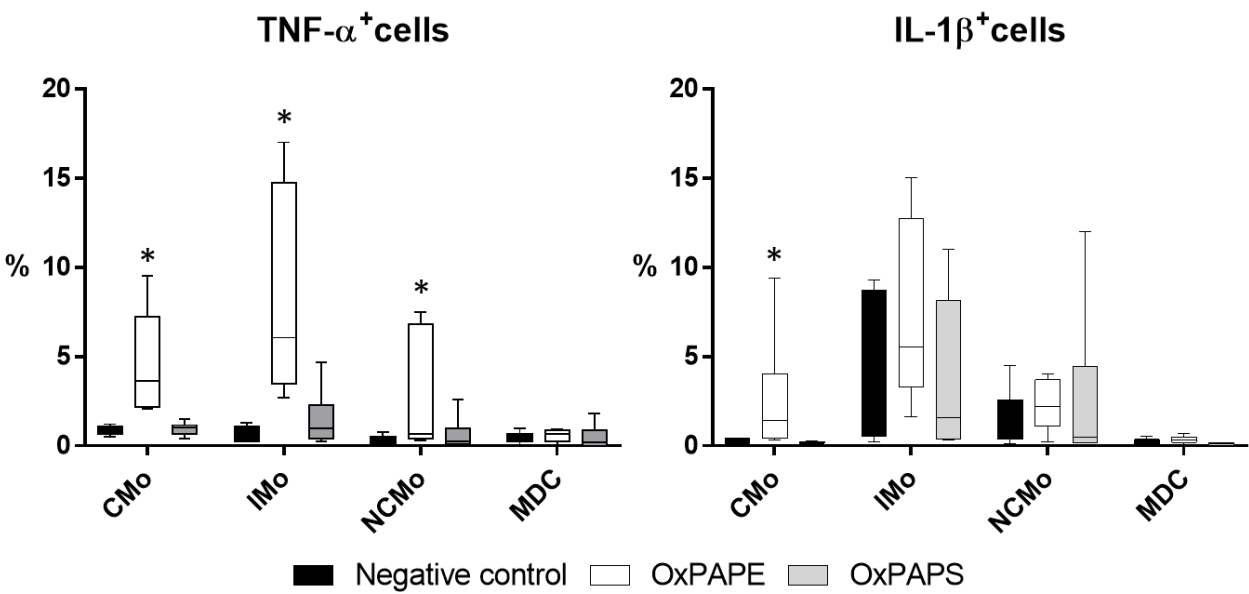
613 **Table 1. Pro-inflammatory effect of OxPAPE.** Frequency of cells producing TNF- α and IL-1 β ,
 614 and amount of cytokine expressed per cell (measured as MFI), among classical (CMo), intermediate
 615 (IMo) and non-classical (NCMo) monocytes, and mDC, under the following culture conditions: in
 616 absence of stimulus (negative control) and after incubation with OxPAPE or OxPAPS. Results are
 617 expressed as a mean \pm standard deviation. Differences with respect to negative control were
 618 considered statistically significant (*) when $p < 0.05$ (paired-sample Wilcoxon test). %, the
 619 percentage of positive cells. MFI, mean fluorescence intensity.

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 621 Frequency of cells producing TNF- α and IL-1 β , and amount of cytokine expressed per cell
 622 (measured as MFI), among classical (CMo), intermediate (IMo) and non-classical (NCMo)

monocytes, and mDC, under the following culture conditions: after LPS-induced inflammatory stimulus (positive control), after incubation with LPS + OxPAPE, or LPS + OxPAPS. Results are expressed as a mean \pm standard deviation. Differences with respect to positive control were considered statistically (*) significant when $p < 0.05$ (paired-sample Wilcoxon test). %, the percentage of positive cells; MFI, mean fluorescence intensity.

		TNF- α		IL-1 β	
		%	MFI	%	MFI
CMo	LPS	99 \pm 0.52	60230 \pm 24783	84 \pm 11	678 \pm 208
	LPS + OxPAPE	100 \pm 0.55	40348 \pm 10770 *	88 \pm 10	541 \pm 147
	LPS + OxPAPS	98 \pm 1.87	31426 \pm 9655 *	75 \pm 27.94	471 \pm 100
IMo	LPS	100 \pm 0.82	114688 \pm 37431	95 \pm 3.20	1434 \pm 915
	LPS + OxPAPE	100 \pm 0.00	85979 \pm 24198	97 \pm 2.97	992 \pm 430
	LPS + OxPAPS	100 \pm 0.82	77434 \pm 30211	87 \pm 16.96	918 \pm 596
NCMo	LPS	99 \pm 1.17	110415 \pm 46131	78 \pm 19	1040 \pm 484
	LPS + OxPAPE	99 \pm 0.55	186478 \pm 226387	79 \pm 10	560 \pm 215
	LPS + OxPAPS	98 \pm 2.48	69449 \pm 20662 *	77 \pm 10.39	697 \pm 389 *
mDC	LPS	82 \pm 12	19392 \pm 10727	53 \pm 21	418 \pm 129
	LPS + OxPAPE	68 \pm 16	17388 \pm 13001	55 \pm 21	381 \pm 93
	LPS + OxPAPS	47 \pm 12 *	15823 \pm 9547	66 \pm 22	327 \pm 80 *

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633 **Figure 1. Pro-inflammatory effect of OxPAPE.** Frequency of cells producing TNF- α and IL-1 β ,
634 among classical (CMo), intermediate (IMo) and non-classical (NCMo) monocytes, and mDC, in the
635 absence of LPS (negative control) and after incubation with OxPAPE and OxPAPS. Differences
636 with respect to negative control were considered statistically significant (*) when $p < 0.05$ (paired-
637 sample Wilcoxon test). %, the percentage of positive cells.

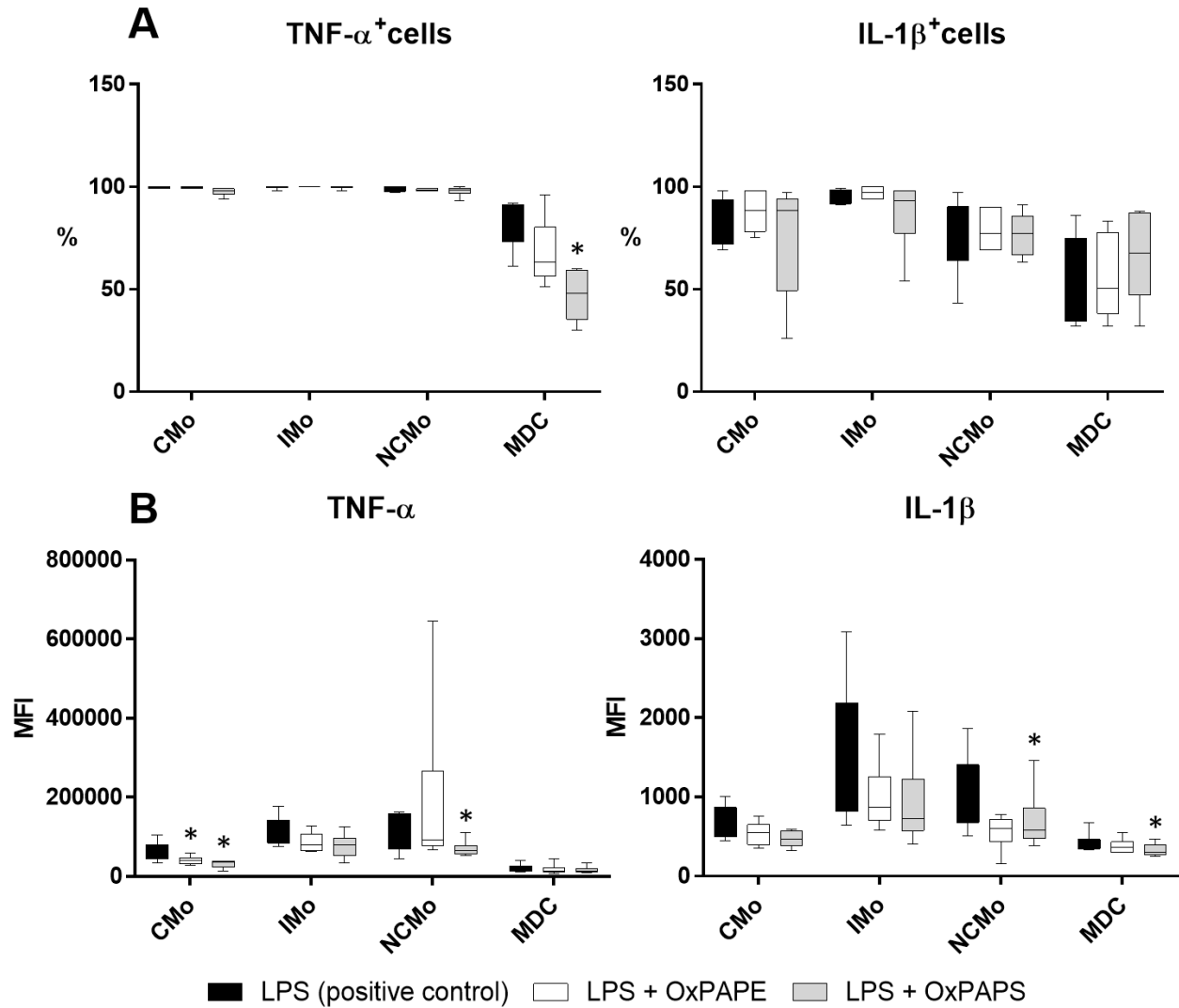


Figure 2. Suppression of the inflammatory effect induced by LPS mediated by OxPAPS.

Frequency of cells producing TNF- α and IL-1 β , among classical (CMo), intermediate (IMo) and non-classical (NCMo) monocytes, and mDC, after stimulation with LPS (positive control), LPS + OxPAPE, or LPS + OxPAPS (A). Amount of TNF- α and IL-1 β produced per cell (measured by MFI), after stimulation with LPS (positive control), LPS + OxPAPE, or LPS + OxPAPS (B). Differences with respect to positive control were considered statistically significant (*) when $p < 0.05$ (paired-sample Wilcoxon test). %, the percentage of positive cells. MFI, mean fluorescence intensity.

648 **Table 1. Pro-inflammatory effect of OxPAPE.** Frequency of cells producing TNF- α and IL-1 β ,
649 and amount of cytokine expressed per cell (measured as MFI), among classical (CMo), intermediate
650 (IMo) and non-classical (NCMo) monocytes, and mDC, under the following culture conditions: in
651 absence of stimulus (negative control) and after incubation with OxPAPE or OxPAPS. Results are
652 expressed as a mean \pm standard deviation. Differences with respect to negative control were
653 considered statistically significant (*) when $p < 0.05$ (paired-sample Wilcoxon test). %, the
654 percentage of positive cells. MFI, mean fluorescence intensity.

		TNF- α		IL-1 β	
		%	MFI	%	MFI
CMo	Negative control	0.81 \pm 0.25	5231 \pm 2332	0.23 \pm 0.15	N/A
	OxPAPE	4.58 \pm 2.89 *	3231 \pm 1399	2.54 \pm 3.43 *	362 \pm 36
	OxPAPS	0.95 \pm 0.36	6103 \pm 2047	0.18 \pm 0.06	586 \pm 315
IMo	Negative control	0.66 \pm 0.43	N/A	4.23 \pm 4.15	N/A
	OxPAPE	8.25 \pm 5.97 *	3214 \pm 1956	7.26 \pm 5.15	375 \pm 84
	OxPAPS	1.46 \pm 1.64	N/A	3.68 \pm 4.41	306 \pm 25
NCMo	Negative control	0.35 \pm 0.25	N/A	1.52 \pm 1.61	N/A
	OxPAPE	2.69 \pm 3.37 *	N/A	2.26 \pm 1.40	336 \pm 36
	OxPAPS	0.60 \pm 0.99	N/A	2.55 \pm 4.68	324 \pm 7.07
mDC	Negative control	0.45 \pm 0.31	N/A	0.17 \pm 0.21	N/A
	OxPAPE	0.58 \pm 0.36	N/A	0.33 \pm 0.23	N/A
	OxPAPS	0.46 \pm 0.68	N/A	0.10 \pm 0.08	N/A

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Table 2. Suppression of the inflammatory effect induced by LPS mediated by OxPAPS.

Frequency of cells producing TNF- α and IL-1 β , and amount of cytokine expressed per cell (measured as MFI), among classical (CMo), intermediate (IMo) and non-classical (NCMo) monocytes, and mDC, under the following culture conditions: after LPS-induced inflammatory stimulus (positive control), after incubation with LPS + OxPAPE, or LPS + OxPAPS. Results are expressed as a mean \pm standard deviation. Differences with respect to positive control were considered statistically (*) significant when $p < 0.05$ (paired-sample Wilcoxon test). %, the percentage of positive cells; MFI, mean fluorescence intensity.

		TNF- α		IL-1 β	
		%	MFI	%	MFI
CMo	LPS	99 \pm 0.52	60230 \pm 24783	84 \pm 11	678 \pm 208
	LPS + OxPAPE	100 \pm 0.55	40348 \pm 10770 *	88 \pm 10	541 \pm 147
	LPS + OxPAPS	98 \pm 1.87	31426 \pm 9655 *	75 \pm 27.94	471 \pm 100
IMo	LPS	100 \pm 0.82	114688 \pm 37431	95 \pm 3.20	1434 \pm 915
	LPS + OxPAPE	100 \pm 0.00	85979 \pm 24198	97 \pm 2.97	992 \pm 430
	LPS + OxPAPS	100 \pm 0.82	77434 \pm 30211	87 \pm 16.96	918 \pm 596
NCMo	LPS	99 \pm 1.17	110415 \pm 46131	78 \pm 19	1040 \pm 484
	LPS + OxPAPE	99 \pm 0.55	186478 \pm 226387	79 \pm 10	560 \pm 215
	LPS + OxPAPS	98 \pm 2.48	69449 \pm 20662 *	77 \pm 10.39	697 \pm 389 *
mDC	LPS	82 \pm 12	19392 \pm 10727	53 \pm 21	418 \pm 129
	LPS + OxPAPE	68 \pm 16	17388 \pm 13001	55 \pm 21	381 \pm 93
	LPS + OxPAPS	47 \pm 12 *	15823 \pm 9547	66 \pm 22	327 \pm 80 *